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14. ABSTRACT
It is hypothesized that the gene encoding RASGRF1, a GTP exchange factor (GEF), controls the severity of neurofibromatosis. **Over-expression of *Rasgrf1* is predicted to exacerbate neurofibromatosis while *Rasgrf1* silencing will attenuate it.** Two novel strains of mice ideally suited to test this hypothesis that were developed in my lab are being used to evaluate the role or *Rasgrf1* on the manifestations of neurofibromatosis type 1. One strain of mice over-express *Rasgrf1*, the other has diminished expression. These were crossed with a mouse model for NF1 and the effects of the altered level of RASGRF1 on tumorigenesis were monitored. The results indicate that over-expression of *Rasgrf1* significantly hastens the time of tumor onset and increases the overall frequency of tumor incidence. In contrast, diminished expression modestly delays the timing of tumor development, but overall frequency of tumor development is not changed. To extend this work from the genetic to biochemical, we have established assays to evaluate Ras activation status. The analysis of tumors and tissues from mice is ongoing. To extend this work further from mouse to human, we are establishing the assays to detect human *RASGRF1* mRNA from human tumor sections using *in situ* hybridization.

15. SUBJECT TERMS
RASGRF1, exchange factor, RAS activation, p53, NF1, mouse model

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INTRODUCTION:

The goal of this research is to expand the knowledge of the genes that contribute to neurofibromatosis beyond the GAP1-related (GTPase activation protein-related) domain in the NF1 (neurofibromatosis 1) protein. It is hypothesized that the gene encoding RASGRF1, a GTP exchange factor (GEF), is one of these genes. **Over-expression of *Rasgrf1* is predicted to exacerbate neurofibromatosis while *Rasgrf1* silencing will attenuate it.** Two novel strains of mice ideally suited to test this hypothesis that were developed in my lab are being used to evaluate the role or *Rasgrf1* on the manisfestations of neurofibromatosis type 1. Please note that a progress report was submitted in December of 2004. This contained information that should have been submitted with this progress report. Therefore, this report contains information included in the December 2004 report as well as additional data.

BODY:

A component of our work has been to evaluate in greater detail and at the molecular level and whole animal level the specific changes in gene expression, signaling events and other phenotypes possibly related to neurofibromatosis phenotypes caused by the various *Rasgrf1* mutations we have generated. Specifically, we have done the work listed in the bullets below using mice harboring the various *Rasgrf1* alleles we have created and crossed with NF1 and NPcis mice. The detailed results follow the bullets.

- Quantify precisely the changes in *Rasgrf1* mRNA production and regions of production.
- Measure the amounts of Ras protein made and assay the extent of Ras activation
- Monitor behavioral phenotypes associated with altered *Rasgrf1* expression

<u>Assays for *Rasgrf1* mRNA</u> To identify region in the brain expressing *Rasgrf1* and to determine if there are differences in *Rasgrf1* expression in the different regions of mouse brain, we performed RT-PCR analysis using RNA taken from the cerebrum, cerebellum and hippocampus. This assay was semiquantitative, using a five fold dilution series of cDNA prepared from these three sites. The results shown in figure 1 reveal that three are no large differences, by brain region, in *Rasgrf1* mRNA expression.

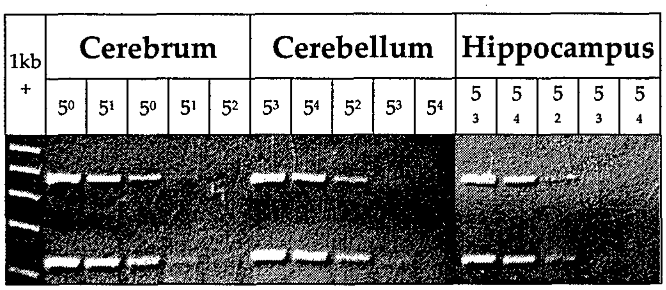

Figure 1. *Rasgrf1* RNA expression levels in the brain. A dilution series of cDNAs prepared from mRNA isolated from different brain regions of wild type (+/+) mice expressing *Rasgrf1* from the paternal allele was amplified by PCR and run on an agarose gel. Primers used detected *Rasgrf1* (top band) and *Rpl32* which was used as a loading control. No large differences were noted for the regions in which *Rasgrf1* is expressed, it appeared at comparable levels in each region tested.

To quantify more precisely the levels of *Rasgrf1* RNA made by our various mutant mice, we performed real time PCR assays. RNA was isolated from whole brains of mutant mice at different developmental stages, cDNAs prepared and real time PCR performed using TaqMan probes for fluorescence detection. The graphs in figure 2 show the results with the Y axis reporting the C_T values of the real time PCR which reflect the relative abundance of *Rasgrf1* RNAs from the mice. Note that high C_T values reflected low mRNA abundance and for every unit difference in C_T value, there is a two fold difference in mRNA abundance. BJR2/+ mice are the animals with biallelic expression of *Rasgrf1*, +/+ are wild type mice with paternal allele-specific expression and +/BJR3 mice lack expression from the single normally active paternal allele in neonates. The data show that levels of *Rasgrf1* RNA are only slightly higher in BJR2/+ than in +/+ neonates, despite the biallelic expression in the former while level in +/BJR3, the *Rasgrf1* RNA level is approximately 5% of that seen in the other two strains of mice. In adult brain, BJR2/+ mice had the highest level of *Rasgrf1* expression which was twice that seen in +/+ animals which was in turn twice that seen in +/BJR3 mice.

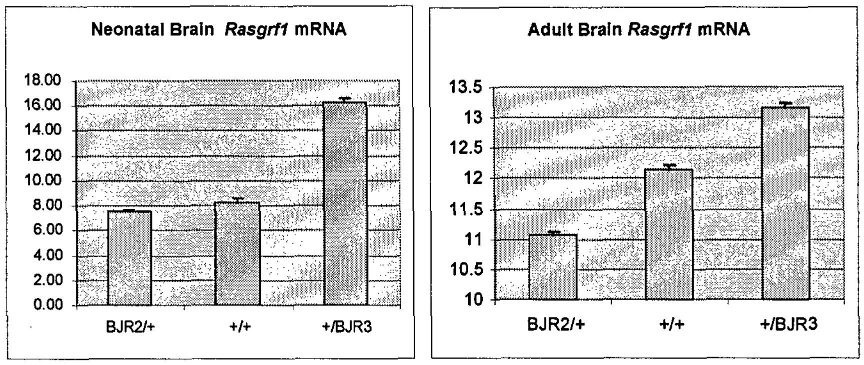

Figure 2. Real time PCR results for assays of *Rasgrf1* mRNA from mice. The y-axis shows Ct values.

***<u>In situ</u>* <u>assays for *Rasgrf1* mRNA</u>** What the RT-PCR analysis does not reveal about *Rasgrf1* expression are the specific cell types that express *Rasgrf1*. This is true even for assays using dissected tissues. To make these measurements, I originally proposed performing *in situ* analysis of *Rasgrf1* RNA. There are two reasons for modifying the approach to do this by immunohistochemistry for RASGRF1 protein: First, protein based assays provide a better measure of functional products from *Rasgrf1* expression. While RNA may be made and reliably detected, any control of mRNA translation may prevent accumulation of protein. In this case, RNA based detection methods may not reflect active protein in the tissue expressing RNA. Second, since the original submission of the grant application, there have been several new commercial sources for antibody that can detect RASGRF1 in paraffin embedded sections. These were not previously available. We are currently in the midst of testing conditions for optimizing detection of RASGRF1 protein in our tissue samples. The variables we are testing focus on means for antigen retrieval using microwave heating and proteinase treatments. Data currently in hand have insufficient signals to show up in images captured and printed on paper. We are still in the early stages of optimization.

<u>Assay for Ras activation status and amount of Ras protein</u> Because there are differences in the amount of expression in the brains of mice of the GTP exchange factor *Rasgrf1*, we wished to measure the extent of Ras activation as measured by the GTP-bound form of Ras. This was measured by a pull down assay using Raf-bound agarose beads – Raf preferentially binds Ras when it is in its GTP-bound state. The pulled down material was detected by Western blot and as a control for loading, the total amount of Ras in the input sample before pull down was measured as well. The results shown in figure 3 reveal no differences in Ras activation status or total Ras levels among +/+, BJR2/+ or +/BJR3 mice. This begs the question "what other signaling

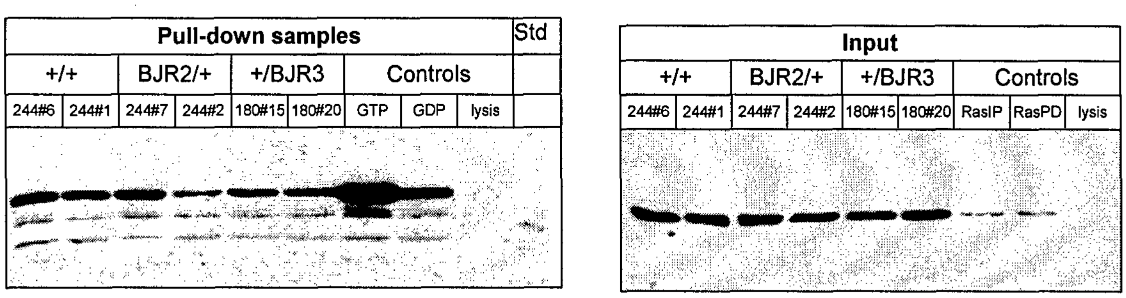

Figure 3. Ras activation status in +/+ (normal expression), BJR2/+ (slight over expression) and +/BJR3 (no expression) mice. Whole brain lysates from duplicate brains of mice were subjected to Raf-mediated pull down of Ras-GTP followed by Western blot analysis of Ras protein (left). The identifying numbers of each mouse are indicated. Controls included lysis buffer alone, or +/+ extracts incubated with GTP or GDP which demonstrated GTP incubation could enhance the activation status of Ras. Total lysates of each sample prior to pull down were also assayed by Western blot (right) for each mouse tested which revealed no significant differences among the mice. Controls included Ras protein that had been isolated by immune precipitation (IP) and also by Raf pull down (PD). Lysis buffer alone was included as a negative control.

events may be modified by *Rasgrf1* expression that may cause the reduced survival in BJR2/+ mice shown in figure 1 of our December 2004 report?" Ongoing assays are focusing on changes in other signaling pathways, Rac in particular. The samples subjected to Ras signaling tests are being analyzed for changes in Rac signaling.

Behavioral assays One of the consequences of neurofibromatosis is altered mental capacity in affected individuals. We have begun some pilot experiments using our mouse model to evaluate how changes in *Rasgrf1* expression may alter cognitive functions that are revealed in an assay for learning. The paradigm involved training neonatal pups with altered *Rasgrf1* expression to recognize an odorant associated with their mother, then testing their ability to move towards the mother-associated odor after being separated from their mother and wish to nurse. Controls were done to ensure there were no inherent locomotion defects or odor preferences for the genotypes. The results show that deficiency in *Rasgrf1* expression impaired the ability of pups to recognize odors associated with their mothers, while animals over expressing *Rasgrf1* had an improved capacity to identify maternal odors (figure 4). We are now testing adult cognitive functions and attempting to frame these phenotypes in the context of any involvement of *Rasgrf1* in phenotypes associated with neurofibromatosis.

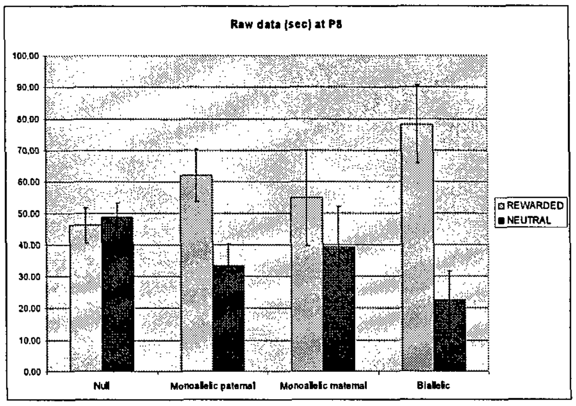

Figure 4. Impaired olfaction-based learning and behavior in *Rasgrf1*-deficient mice and improved responses in *Rasgrf1* over-expressing mice. Five day old mice were separated from their mothers for one hour, during which time the mothers were scented with an odorant (rewarded odor). Upon return of pups to their mothers, they nursed and could detect the rewarded odor. At 8 days of age, mice were separated from the mothers for another hour then tested in a chamber that presented the rewarded odor and a related but distinct neutral odor. The time, in seconds, spent in each sector of the chamber where the odors were concentrated was measured. Mice used were "null" (labeled +/BJR3 in figure 2) which are deficient for *Rasgrf1* ; "monoallelic maternal" which have normal levels of *Ragrf1* expression which is from the maternal allele; "monoallelic paternal" (labeled +/+ in figure 2) which have normal levels of *Ragrf1* expression which is from the paternal allele; or "biallelic" (labeled BJR2/+ in figure 2) which slightly over express *Rasgrf1*.

KEY RESEARCH ACCOMPLISHMENTS:

- ☐ Identified three regions in the mouse brain expressing *Rasgrf1*.
- ☐ Quantified changes in *Rasgrf1* expression in mutant and wild type mice at different developmental stages by real time PCR.
- ☐ Completed assays for Ras activation status in brains of wild type and mutant mice, initiated assays for Rac activation status.
- ☐ Began immunohistochemsitry based assays for RASGRF1 protein in our tissue samples.
- ☐ Documented learning defects in a model of RASGRF1 deficiency.

REPORTABLE OUTCOMES:

Additional analysis of collected specimens is needed prior to reporting.

CONCLUSIONS:

Molecular mechanisms by which *Rasgrf1* over expression may influence tumor development, previously reported, remain elusive. Our hypothesis that changes in Ras activation status are the key were not bourn out by our assays for GTP-bound Ras. Additional assays for other intracellular signaling events affected by *Rasgrf1* are warranted and in progress. A role for *Rasgrf1* has been found in behavioral responses to olfaction-based learning and responses.